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Commonly Used Disinfectants Fail To Eradicate *Salmonella enterica* Biofilms from Food Contact Surface Materials

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Salmonellosis is the second most common cause of food-borne illness worldwide. Contamination of surfaces in food processing environments may result in biofilm formation with a risk of food contamination. Effective decontamination of biofilm-contaminated surfaces is challenging. Using the CDC biofilm reactor, the activities of sodium hypochlorite, sodium hydroxide, and benzalkonium chloride were examined against an early (48-h) and relatively mature (168-h) *Salmonella* biofilm. All 3 agents result in reduction in viable counts of *Salmonella*; however, only sodium hydroxide resulted in eradication of the early biofilm. None of the agents achieved eradication of mature biofilm, even at the 90-min contact time. Studies of activity of chemical disinfection against biofilm should include assessment of activity against mature biofilm. The difficulty of eradication of established *Salmonella* biofilm serves to emphasize the priority of preventing access of *Salmonella* to postcook areas of food production facilities.

Salmonella species have been described as environmental persisters (1). Previously published reports have suggested that *Salmonella* can remain on surfaces and equipment used for handling and washing raw meat and that conventional cleaning and sanitation may fail to eradicate *Salmonella* from such surfaces (2, 3). Molecular typing has indicated that particular strains of *Salmonella* can persist for up to 10 years in food processing environments (4). Russo et al. recently reported that a *Salmonella enterica* subsp. *enterica* serovar Agona strain responsible for two food-borne outbreaks remained in the environment of a food processing facility for 10 years despite intensive cleaning and decommissioning of contaminated equipment (5). *S.* Agona has also previously been linked to recurrent food-borne outbreaks (5–9). In the context of a major international outbreak of *S.* Agona in 2008 (10), it was of interest to assess the ability of chemical disinfectants to control *Salmonella* persistence on food contact surfaces.

Persistence of *Salmonella* in food processing environments after cleaning and sanitation may be related to inadequate processes. However, even in the context of well-controlled processes, acquired disinfectant resistance may be a consideration. Over an extended period of exposure, or through repeated intermittent exposure, *Salmonella* can develop such acquired resistance (11, 12). Furthermore, even *Salmonella* species that remain susceptible to inactivation when in planktonic phase may be much more resistant in a biofilm phase. *Salmonella* readily forms a biofilm on food contact surfaces in both industrial and domestic settings (13–16). Thus, biofilm formation is likely to be relevant to long-term persistence of *Salmonella* on surfaces. *Salmonella* biofilm may act as a reservoir for recurrent bacterial contamination in a food processing facility, which may lead to multiple food-borne outbreaks.

Salmonella biofilm growth has been examined through laboratory-based models on a diverse range of surfaces, including concrete, tile, stainless steel, glass, silestone, granite, rubber, and synthetic plastics (16, 17). Surface properties such as crevices and pitting after abrasive cleaning may contribute to increased bacterial attachment, biofilm formation, and resistance to disinfectant agents (18, 19).

A number of authors suggest that biofilm formed over an extensive period of time has increased resistance to antimicrobial substances (20–26), while others have reported that the age of the biofilm does not enhance resistance to disinfectants (27). Increased extrapolymeric substances and biofilm thickness over time are reported by some to enhance resistance to disinfectant agents (18, 28).

Lianou and Koutsoumanis recently argued that it was vital to assess *S. enterica* biofilm properties on multiple strains of the pathogen (29). However, a number of authors have described *Salmonella* biofilm formation with a limited number of strains (30, 31), with a single serovar (16, 18, 26, 32–37), or by using a single biofilm substratum (29, 38–41). There have also been significant studies in this regard related to other genera of bacteria, notably *Listeria monocytogenes* (42–44). However, the existing literature does not yield a consistent picture of the activity of disinfectant against *Salmonella* biofilm, and there is similar uncertainty regarding the impact of age on biofilm properties. The objective of this paper is to clarify these issues for *Salmonella enterica* using the CDC biofilm reactor (CBR) model, with methods and strains previously characterized in detail with respect to biofilm formation at 48 h on multiple surfaces. We have previously reported a detailed quantitative characterization of biofilm formation at 48 h by these strains on a variety of surfaces in the CBR model, including a definition of the limits of repeatability (45). In this paper, we assess biofilm density at 168 h compared with that at 48 h on multiple surfaces and the activity of commonly used chemical disinfectants against early (48-h) and more mature (168-h) *Salmonella* biofilms for a subset of those strains representing common serovars on a concrete substratum. The chosen concentrations were

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based on a review of the concentrations used in industry and comparable to those used in other work published in this area (46–49). These concentrations were also demonstrated to be effective against planktonic cells in preliminary experiments described in this work.

MATERIALS AND METHODS

Activity of disinfectants against planktonic cells. Each test strain was stored on cryogenic glycerol beads (Protect Beads; Technical Services, Lancashire, United Kingdom) and recovered on tryptic soy agar (TSA) plates (Sigma-Aldrich Ireland Limited, Arklow, Ireland). Colonies were suspended in phosphate-buffered saline (PBS) (Sigma-Aldrich) to a 0.5 McFarland suspension (~1.5×10^8 CFU/ml). Fresh solutions of disinfectants were used for each experiment. A preliminary MIC was performed similar to the ISO 20766-1 method (50). One milliliter of the 0.5 McFarland standard suspension was diluted into 9 ml of tryptic soy broth (TSB; Sigma-Aldrich) and vortexed. One hundred microliters of the suspension was then added in triplicate to wells of a 96-well round-bottom microtiter plate (Sarstedt AG & Company, Nümbrecht, Germany). The MIC was determined twice on different days. The stock concentrations of sodium hypochlorite (2,000 mg/liter), sodium hydroxide (4 M concentration), and benzalkonium chloride (0.08%) (Sigma-Aldrich) disinfectants were made before each test. The stock concentrations were serially diluted in sterile water. For testing the activity of the disinfectants against planktonic cells, a broad range of concentrations were achieved by serial dilutions. A 100-μl aliquot of each disinfectant concentration was added to 100 μl of bacterial suspension in a microtiter tray. The plates were incubated for 24 h at 37°C. The plates were examined visually to assess turbidity.

CDC biofilm reactor. To establish biofilm, 1 ml of the suspension was diluted into 9 ml of tryptic soy broth (Sigma-Aldrich) and incubated at 37°C for 24 h in a shaking incubator. One milliliter of this bacterial suspension was used to inoculate the reactor vessel (CBR; model CBR 90-2; BioSurface Technologies, Bozeman, MT) by following the method previously described (45). The CBR contained 20 standard sterile coupons (1.25-cm diameter, 0.3-cm depth), 4 each of glazed tiles (RD128-GT), 316L stainless steel (RD128-316), borosilicate glass (RD128-GL), polycarbonate plastic (RD128-PC), and concrete (RD128-CC) coupons supplied by BioSurface Technologies. The CBR was operated under batch phase for 24 h using full-strength TSB followed by continuous-flow phase for 24 h (standardized 48-h biofilm) or operated for an additional 144 h (extended 7-day biofilm) using 10 g/liter TSB (Sigma-Aldrich Ireland Limited, Arklow, Ireland). All experiments were performed at room temperature. After each run (48 h/168 h), the CBR was aseptically dismantled. Coupons were washed with sterile water to remove loosely adherent planktonic cells and then placed into 10-ml capped universals with sterile PBS (Sigma-Aldrich) and subjected to sonication at 20 kHz for 7 min (ULTRASonik model 28×; Ney Dental, Inc., Bloomfield, CT) followed by vortexing at high speed (Vortex model 100-2400; VWR International, Germany). The disaggregated biofilm was serially diluted for viable counts by the spread plate technique.

The biofilm was assessed for all strains on 3 separate runs (48-h biofilm) or on 2 separate runs (168-h biofilm) in the CBR, and on each occasion enumeration was performed on 3 coupons of each material. This resulted in 9 (3 coupons × 3 runs) or 6 (3 coupons × 2 runs) counts used for statistical analysis. The disinfectant tests were performed in duplicate. Based on the sonication settings examined, 7-min sonication at 20 kHz resulted in a greater number of cells recovered from surfaces than sonication for a longer period of time (10, 14 min) or increased sonication power (25, 50 kHz).

SEM. Coupons were prepared for scanning electron microscopy (SEM) by primary fixation in a glutaraldehyde-paraformaldehyde solution (Sigma-Aldrich), secondary fixation in osmium tetroxide (Agar Scientific, Essex, United Kingdom), and dehydration in an ethanol series as previously described (45). The coupons were dried using hexamethyl disilazane (Agar Scientific). The samples were sputter-coated with gold (Emscope, SC500) and examined with SEM (Hitachi S-2600N).

Activity of disinfectants against biofilm cells. Fresh working solutions of the disinfectants were made directly before each experiment. A single concentration of each disinfectant (sodium hypochlorite, 500 mg/liter; sodium hydroxide, 1 M; and benzalkonium chloride, 0.02%) was selected for testing against an established biofilm on concrete surfaces for 10, 45, and 90 min. Difco D/E neutralizing broth (39 g/liter) (Becton Dickenson, Oxford, United Kingdom) was used to neutralize sodium hydroxide and benzalkonium chloride, and sodium thiosulfate (11.2 g/liter) (Sigma-Aldrich) was used to neutralize sodium hypochlorite (contact time, 30 min). Two CBRs were used as described above. Following establishment of the biofilm, the concrete coupons were released from the CBRs and aseptically placed into individual wells of a 24-well microtiter plate (Sarstedt). For each disinfectant studied, the coupons in 2 wells were covered with 1 ml of 1 M sodium hydroxide, 500 mg/liter of sodium hypochlorite, 0.02% benzalkonium chloride, or 1 ml of sterile PBS (negative control). After the specified contact time, the coupons were immersed in the neutralizing agents for 30 min and then aseptically placed in capped glass universal containers with 10 ml of PBS. The coupons were then sonicated, vortexed, serially diluted, and spread onto TSA as previously described. The colonies were counted to determine the mean $\log_{10}$ density of cells recovered. The 2nd coupon was entirely immersed in 10 ml TSB and incubated overnight at 37°C. Following overnight incubation, the turbidity of the broth was examined to assess regrowth from the coupon. Turbid broths were subcultured to confirm *Salmonella* (based on typical morphology).

Mean log density was calculated using a formula previously described (45). Statistical analysis was performed using SPSS version 20 using a Mann-Whitney *U* test for nonparametric data. The $\log_{10}$ reduction of cells recovered from the surface after contact was calculated as previously described in the CEN standards (E13697:2001).

RESULTS

Difference between the standard 48-h biofilm and the extended 168-h biofilm. SEM indicated that there were morphological differences between the biofilm formed at 48 h (Fig. 1A) and the biofilm formed by the same *S.* Agona strain at 168 h (Fig. 1B). However, the SEM evaluation of removal of 168-h biofilm from coupons by sonication indicated incomplete removal of biofilm (as displayed in Fig. 1C). This was in contrast to the 48-h biofilm for these strains and surfaces, with the exception of *S. enterica* serovar Typhimurium LT2 (data not shown). It was not possible to develop a modification of sonication conditions that achieved complete removal of biofilm from the coupon without impacting on viability of cells. Therefore, estimates of 168-h biofilm density presented here and based on enumeration of recovered cells underestimate the true density of the 168-h biofilm.

Table 1 presents the density of *S. enterica* (mean $\log_{10}$ CFU/coupon) biofilm at 48 h as previously described (45) compared with that at 168 h. Despite the underestimation of 168-h biofilm density, in general, more cells were recovered from the 168-h biofilm than the 48-h biofilm. As demonstrated in Table 1, this difference is statistically significant on all 5 surfaces for the *S.* Agona outbreak strain (S09-0494), *S.* Typhimurium SL1344, and *S. enterica* serovar Enteritidis S09-0717 ($P \leq 0.05$).

Table 2 demonstrates the interstrain differences between the *S. enterica* strains after 168-h biofilm formation. The *S. enterica* serovar Agona outbreak strain (S09-0494) formed a more dense biofilm than the *S.* Agona SL483 strain on all 5 surfaces ($P = 0.004$) and *S.* Typhimurium SL1344 and S09-0419 on most surfaces. The *S.* Enteritidis strain (S09-0717) also formed a more

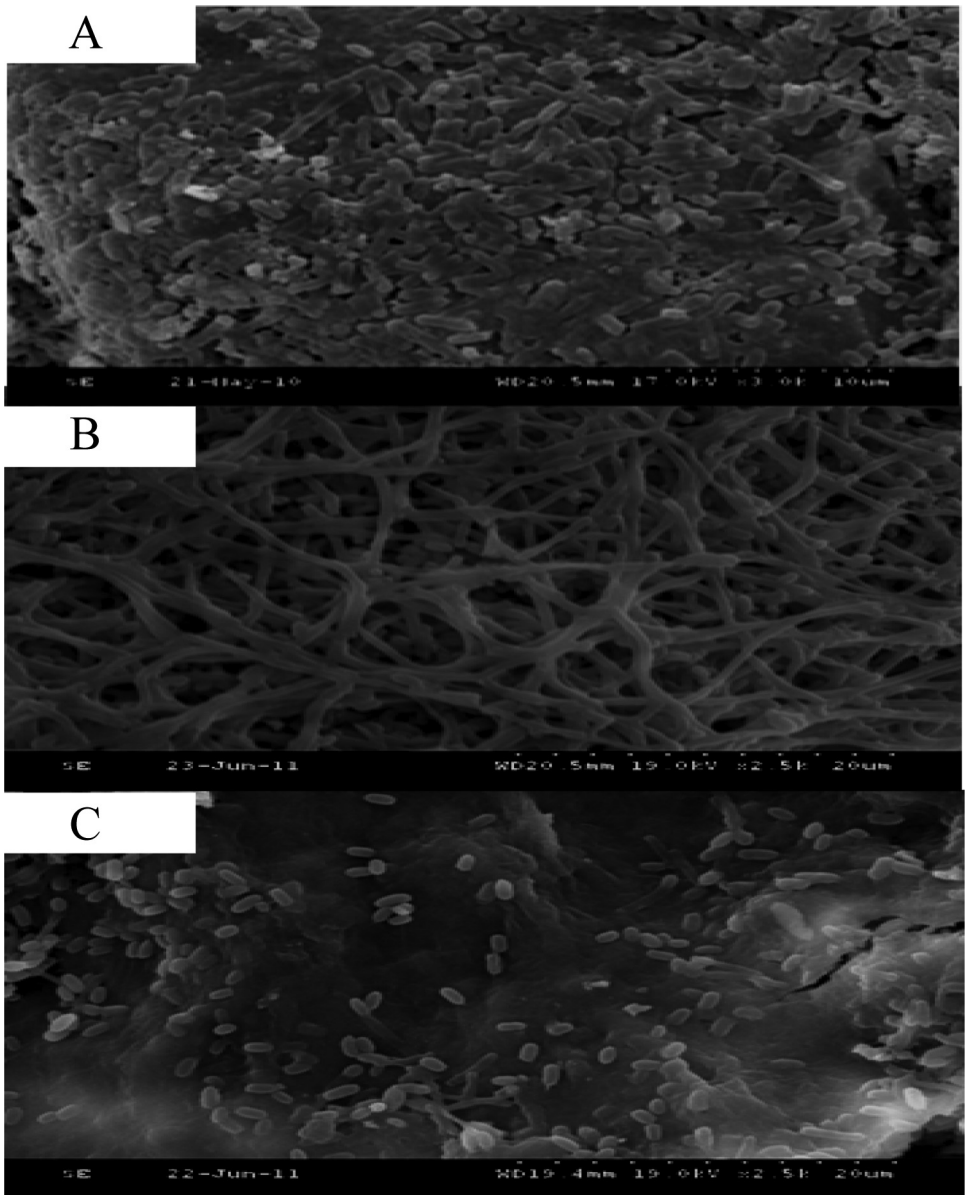

FIG 1 SEM image of biofilm remaining on surface postsonication displays *Salmonella* Agona biofilm on a concrete coupon after 48 h (A) and 168 h (B) of biofilm development. Panel C displays the 168-h biofilm after 7 min of sonication. Images in Fig. 1 were taken using scanning electron microscopy under high magnification (×2,500).

dense biofilm than the two *S.* Typhimurium strains ($P < 0.05$ on 4 of the 5 surfaces).

Efficacy of disinfectants against planktonic cells and an established biofilm. Using a series of concentrations to assess activity against planktonic cells, we found that concentrations of 0.5 M sodium hydroxide, 250 mg/liter sodium hypochlorite, and 0.01% benzalkonium chloride were sufficient to inhibit growth of planktonic cells for all strains studied under conditions modeled on the ISO 20766-1 standard used to determine the MIC. Therefore, the single fixed concentration chosen for each disinfectant to assess activity against biofilm was in each case 2-fold higher than the MIC for planktonic cells.

With respect to the 48-h biofilm (Table 3), there was a trend toward a reduction in cell counts with longer exposure to all disinfectants (up to 90 min). However, using a concentration 2-fold higher than the lowest concentration that achieved complete inhibition of growth of planktonic cells, these concentrations of sodium hypochlorite and benzalkonium chloride were not effective against an established biofilm. The results demonstrate that only sodium hydroxide eliminated all cells from the 48-h biofilm (Table 4). With respect to the 168-h biofilm, no disinfectant achieved complete eradication of *Salmonella* or a $\geq 4 \log_{10}$ reduction (as per the CEN standard EN13697:2001).

DISCUSSION

When *Salmonella* becomes established in food processing environments, its tendency to persist in the face of cleaning and chem-

TABLE 1 Difference between the 48-h and the 168-h biofilm[a]

Strain	No. of counts	Log_{10} density of cells																			
		Glass				Steel				Polycarbonate				Concrete				Tile			
		168 h	48 h	Diff.	*P*	168 h	48 h	Diff.	*P*	168 h	48 h	Diff.	*P*	168 h	48 h	Diff.	*P*	168 h	48 h	Diff.	*P*
S09-0494	6 and 9	7.26	5.41	1.85	0.001	6.92	5.80	1.12	0.001	6.67	6.33	0.34	0.011	7.59	7.08	0.51	0.001	7.87	7.56	0.31	0.018
SL483	6 and 9	5.91	5.81	0.10	0.553	5.29	6.12	−0.83	0.007	5.46	5.59	−0.13	0.003	7.08	6.75	0.33	0.033	7.52	6.94	0.58	0.001
S09-0419	6 and 9	6.30	5.41	0.89	0.001	6.00	6.28	−0.28	0.087	6.25	6.53	−0.28	0.260	6.93	7.00	−0.07	0.906	7.73	7.23	0.50	0.001
SL1344	6 and 9	5.97	5.15	0.82	0.015	6.59	5.71	0.88	0.001	6.30	6.10	0.20	0.050	7.47	6.78	0.69	0.001	7.62	7.17	0.45	0.001
S09-0717	6 and 9	6.73	4.85	1.88	0.001	6.87	4.73	2.14	0.001	6.80	5.20	1.60	0.001	7.65	6.43	1.22	0.001	8.04	7.02	1.02	0.001

[a] The mean log_{10} density of cells recovered from the surfaces after 48 and 168 h of biofilm formation. The strains examined included 2 *Salmonella enterica* subsp. *enterica* serovar Agona strains (S09-0494 and SL483), 2 *S.* Typhimurium strains (S09-0419 and SL1344), and 1 *S.* Enteritidis strain (S09-0717). The density was measured in mean log_{10} CFU/coupon. The mean differences (Diff.; 168 h − 48 h mean log_{10} density) and *P* values were determined using the Mann-Whitney *U* test. All experiments were performed in duplicate ($n = 6$ counts) or triplicate ($n = 9$ counts), with three coupons used as technical replicates during each experiment.

ical decontamination is problematic. Biofilm formation is one of the processes that may contribute to *Salmonella* persistence (51).

Mangalappalli-Illathu et al. found that *Salmonella enterica* subsp. *enterica* serovar Enteritidis biofilm thickness increased over time to a peak at 120 to 144 h depending on the reactor conditions (26). This research suggested that the structure of a *Salmonella* biofilm changed over time, possibly in ways that provide additional protections from harsh environmental conditions, such as disinfectant treatment (26). Using a similar glass flow model, Korber et al. found that trisodium phosphate was not as effective at killing *S.* Enteritidis biofilm formed over 72 h in comparison to a biofilm formed by the strain for 48 h (18).

However, Habimana and colleagues found that the density of *Salmonella* biofilm decreased linearly over a period of 28 days using a glass flow cell device (25). The results indicated that the environmental genera *Pseudomonas*, *Staphylococcus*, and *Pantoea* produced a denser biofilm over time and were more tolerant to disinfectant treatment than the *S.* Agona strain. Habimana et al. reported the *S.* Agona cells attached directly to the substrata, while the environmental species formed a superstructure on top of the *S.* Agona cells (25). Therefore, it is possible that the mixed biofilm resulted in enhanced biofilm growth and resistance to disinfectant treatment (25).

Others have also suggested that *S. enterica* biofilm formation over an extended period of time of up to 7 days does not influence the efficacy of disinfectant products (27, 49). However, as suggested elsewhere, the limited surface area of the MBEC system described by Wong et al. (27), which is similar in size to the microtiter plate-based systems, may have resulted in limited biofilm development, as discussed elsewhere (41). This may limit the comparability of results from this method with those from models such as the CBR that provide greater surface area. However, Møretrø et al. also reported that the numbers of colonies released from 2-, 3-, 6-, and 9-day biofilms formed on glass slides were similar for two *S. enterica* strains examined (49). Moreover, there was no apparent difference in the disinfection of an *S. enterica* serovar Senftenberg biofilm formed over the extended time when treated with sodium hypochlorite (49).

Our data indicate that the properties of *S. enterica* biofilm on most surfaces relevant to the food processing environment change over time. Based on SEM images provided in Fig. 1, the 168-h biofilm was morphologically different from the 48-h biofilm. The biofilm was also more adherent to the substratum, as the sonication conditions that completely removed the 48-h biofilm failed to achieve complete removal of the 168-h biofilm. The failure to achieve complete removal of the 168-h biofilm from coupons represents a methodological limitation of the application of viable counts to assessment of biofilm density. This serves to emphasize the need to assess completeness of removal of biofilm in such experiments to ensure that such limitations are apparent (52).

TABLE 2 Interstrain variation of biofilm density at 168 h[a]

Strain	No. of counts	Log_{10} density of cells														
		Glass			Steel			Polycarbonate			Concrete			Tile		
		Mean	Diff.	*P*	Mean	Diff.	*P*	Mean	Diff.	*P*	Mean	Diff.	*P*	Mean	Diff.	*P*
S. Agona S09-0494		**7.26**			**6.92**			**6.67**			**7.59**			**7.87**		
S. Agona SL483	6	5.91	1.35	0.004	5.29	1.63	0.004	5.46	1.21	0.004	7.08	0.51	0.004	7.52	0.35	0.004
S. Typhimurium S09-0419	6	6.30	0.96	0.004	6.00	0.92	0.004	6.25	0.42	0.008	6.93	0.66	0.004	7.73	0.14	0.262
S. Typhimurium SL1344	6	5.97	1.29	0.004	6.59	0.33	0.087	6.30	0.37	0.012	7.47	0.12	0.077	7.62	0.25	0.004
S. Enteritidis S09-0717	6	6.73	0.53	0.128	6.87	0.05	0.81	6.80	−0.13	0.261	7.65	−0.06	0.262	8.04	−0.17	0.043
S. Enteritidis S09-0717	**6**	**6.73**			**6.87**			**6.80**			**7.65**			**8.04**		
S. Agona SL483	6	5.91	0.82	0.037	5.29	1.58	0.004	5.46	1.34	0.004	7.08	0.57	0.004	7.52	0.52	0.004
S. Typhimurium S09-0419	6	6.30	0.43	0.228	6.00	0.87	0.013	6.25	0.55	0.01	6.93	0.72	0.004	7.73	0.31	0.004
S. Typhimurium SL1344	6	5.97	0.76	0.045	6.59	0.28	0.107	6.30	0.50	0.029	7.47	0.18	0.016	7.62	0.42	0.004

[a] The results of interstrain comparisons of *Salmonella enterica*. The mean log_{10} density of cells recovered from *Salmonella enterica* subsp. *enterica* serovar Agona outbreak strain S09-0494 was compared to that of 2 *S.* Typhimurium strains (S09-0419 and SL1344) and *S.* Enteritidis S09-0717. The density was measured in mean log_{10} CFU/coupon. The mean differences and *P* values determined using the Mann-Whitney *U* test are also listed. All experiments were performed in duplicate ($n = 6$ counts), with three coupons used as technical replicates during each experiment. The strain names (and values) in boldface are those to which the strains (and values) listed beneath were compared.

TABLE 3 Mean $\log_{10}$ of cells recovered from the 48-h biofilm after contact with disinfectants[a]

Disinfectant	No. of counts	Time (min)	$\log_{10}$ density of cells					
			SL1344			SL483		
			Mean	LR	SD	Mean	LR	SD
Disinfectant-free control			7.63		0.02	7.66		0.01
Sodium hydroxide, 1 M	6	10	0	7.63	0	0	7.66	0
	6	45	0	7.63	0	0	7.66	0
	6	90	0	7.63	0	0	7.66	0
Sodium hypochlorite, 500 mg/liter	6	10	7.48	0.15	0.03	7.53	0.13	0.13
	6	45	7.45	0.18	0.04	7.36	0.30	0.25
	6	90	6.52	1.11	0.19	7.15	0.51	0.36
Benzalkonium chloride, 0.02%	6	10	7.41	0.22	0.09	7.48	0.18	0.16
	6	45	7.32	0.31	0.01	7.24	0.42	0.34
	6	90	6.80	0.83	0.60	6.69	0.97	0.18

[a] Mean $\log_{10}$ and standard deviations of cells recovered from *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 and *S.* Agona SL483 biofilm attached to concrete coupons at 48 h before and after contact with 3 disinfectants for 10, 45, and 90 min. The density was measured in mean $\log_{10}$ CFU/coupon. The $\log_{10}$ reduction (LR) was calculated by subtracting the mean $\log_{10}$ of cells from the disinfectant-exposed coupon from the control coupon.

This is frequently not verified in the literature, which may contribute in part to inconsistencies regarding changes in biofilm density over time. We also acknowledge that the generally accepted threshold of statistical significance ($P \leq 0.05$) has limitations in a setting of multiple comparisons but note that in many instances the *P* values observed here are very much lower than that minimum threshold for significance. Nevertheless, even with the limitations, it is clear that in the CBR model, the 168-h biofilm is in general denser on all/most surfaces than the 48-h biofilm.

The literature is inconsistent regarding changes in *Salmonella* biofilm over time, with some authors also finding evidence of increasing biofilm density after 48 h (53), but some, such as Wong et al., have suggested that *S. enterica* biofilm density does not increase significantly over time (27). Likewise, Møretrø et al. indicated the numbers of colonies recovered from 2-, 3-, 6-, and 9-day biofilms were similar for the 2 *S. enterica* strains studied (49).

It is particularly interesting to note that at 168 h, the comparatively low density of the *S.* Enteritidis biofilm compared to that of *S.* Agona and *S.* Typhimurium has been eliminated or reversed, in comparison to biofilm formed at 48 h. *S.* Enteritidis has been considered a poor biofilm former compared with *S.* Typhimurium and *S.* Agona; however, our data suggest that it might be more appropriate to consider it as a slow biofilm former. Previous authors have also suggested that *S.* Enteritidis can form a dense biofilm over an extended period of time (18, 26, 34, 36, 53). However, as only 1 *S.* Enteritidis strain was included in this assessment, caution is required.

This work evaluates the efficacy of 3 widely used disinfectants with established activity against planktonic *Salmonella* but which differ greatly in terms of their activity against biofilm in this model. This difference may relate to their different mechanisms of action. Benzalkonium chloride is understood to act by disruption of lipid membrane bilayers (54). Hypochlorite solution is acidic, and it acts to generate oxygen radicals that are bactericidal through nonspecific interaction with organic molecules and is readily inactivated by contact with organic matter (54). Sodium hydroxide solution is strongly alkaline and agents with a mechanism of action that includes disaggregate lipid bilayers and organic

TABLE 4 Mean $\log_{10}$ density of viable 168-h biofilm cells after contact with disinfectants[a]

Disinfectant	No. of counts	Time (min)	$\log_{10}$ density of cells											
			SL1344			SL483			S09-0494			S09-0717		
			Mean	LR	SD	Mean	LR	SD	Mean	LR	SD	Mean	LR	SD
Disinfectant-free control			9.74		0.04	9.78		0.04	9.74		0.03	9.68		0.01
Sodium hydroxide, 1 M	6	10	8.24	1.50	0.24	8.13	1.65	0.38	8.10	1.64	0.36	8.89	0.79	0.19
	6	45	8.01	1.73	0.50	7.96	1.82	0.33	8.13	1.61	0.21	8.88	0.80	0.16
	6	90	7.72	2.02	0.57	7.45	2.33	0.12	7.74	2.00	0.26	8.84	0.84	0.17
Sodium hypochlorite, 500 mg/liter	6	10	9.48	0.26	0.02	9.56	0.22	0.13	9.32	0.42	0.01	9.33	0.35	0.01
	6	45	9.43	0.31	0.07	9.51	0.27	0.13	9.28	0.46	0.07	9.00	0.68	0.25
	6	90	9.40	0.34	0.08	9.50	0.28	0.14	8.77	0.97	0.74	8.89	0.79	0.32
Benzalkonium chloride, 0.02%	6	10	9.68	0.06	0.08	9.74	0.04	0.05	9.71	0.03	0.06	9.58	0.10	0.07
	6	45	9.65	0.09	0.09	9.75	0.03	0.04	9.69	0.05	0.07	9.51	0.17	0.09
	6	90	9.64	0.10	0.09	9.47	0.31	0.24	9.65	0.09	0.12	9.49	0.19	0.09

[a] The mean $\log_{10}$ densities and standard deviations of cells recovered from *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 and *S.* Agona SL483 biofilm attached to concrete coupons at 168 h before and after contact with 3 disinfectants for 10, 45, and 90 min. The density was measured in mean $\log_{10}$ CFU/coupon. The $\log_{10}$ reduction (LR) was calculated by subtracting the mean $\log_{10}$ of cells from the disinfectant-exposed coupon from the control coupon.

matter (55). These differences in mechanisms of action and pH of the solutions are likely to contribute to the observed differences in action against *Salmonella* biofilm. In this context, it may be of interest to determine if other caustic solutions have activity similar to that observed with sodium hydroxide. It seems possible that hypochlorite may not have been able to penetrate effectively through the organic layers of the biofilm, which may have contributed to its lack of effect (56).

It is also important to note that these results are based on static exposure to the three disinfectants and in this regard do not address the potentiation of disinfectant activity associated with abrasive or mechanical cleaning which may be used in real-world settings.

The use of a single substratum, in this case concrete, represents a limitation, as it may not be possible to generalize from one substratum to others. Concrete was chosen in this case, because our previous work demonstrated dense biofilm formation on concrete, and concrete is a very common surface in food processing environments, although generally not in direct contact with food.

As with biofilm density, there are inconsistencies in reported activity of chemical disinfectants against *Salmonella* biofilm. Møretrø and colleagues found that while exposure to acidic peroxygen-based disinfectants (Virkon S) resulted in the 4 $\log_{10}$ reduction in *Salmonella* cells, other disinfectants, including hypochlorite and benzalkonium chloride products, did not show sufficient bactericidal effect (49). Vetsby et al. also concluded that neither hypochlorite nor benzalkonium chloride achieved a >4 $\log_{10}$ reduction, although when combining the disinfectants used with a synthetic furanone, this target was achieved (46). In contrast, Wong et al. found that a range of disinfectants, including benzalkonium chloride and sodium hypochlorite, reduced 3-, 5-, and 7-day *Salmonella* biofilms by >4 $\log_{10}$ (27). However, in many instances, the concentration necessary to reduce the cell numbers above the >4 $\log_{10}$ threshold was also above the concentration recommended by the manufacturer (27). The difference in results may reflect the differences in the method of biofilm formation and in particular the surface area available for biofilm formation, for example, in the MBEC method. Møretrø et al. also highlighted the difference in efficacy of disinfectants using different methods of testing, such as the pellicle test, suspension test, and European surface test EN 13697:2001 (49).

Control of *Salmonella* in food processing environments can be a major challenge. Biofilm formation is one aspect of this problem. The ability to assess likely efficacy of biocides or disinfectants in the eradication of *Salmonella* is therefore of considerable practical importance; however, the available data are problematic at many levels. There are inconsistent findings, often with limited numbers of strains, in diverse models or in some cases with limited data on repeatability. It has been suggested that the CBR provides a more robust measure of the efficacy of disinfectant products against an established biofilm compared with other methods (57). However, even with the CBR, the confidence intervals for measurements of biofilm density are wide. The method is time-consuming and comparatively expensive, particularly with replication to assess repeatability. For the biofilm formed over the extended period of time, we find the method is further limited by difficulties in achieving complete biofilm detachment without cell damage. Given the difficulties of methodology and the inconsistencies in reported results, there is a need for multicenter studies of specific strains using agreed-upon methods to achieve consensus on the properties of *Salmonella* biofilm and, beyond this, to assess activity against mixed-species biofilm intermixed with food residues, which reflects the reality of food processing environments.

In summary, notwithstanding the methodological issues, our data suggest there are substantial differences between 48-h and 168-h biofilms, that sodium hypochlorite and benzalkonium chloride have little efficacy against *Salmonella* biofilm on concrete, and that none of the 3 disinfectants studied are capable, on their own, of eradicating an established 168-h *Salmonella* biofilm. This finding may have implications for use of biocides and disinfectants, because studies on efficacy of biocides have generally used only 48-h biofilm (12, 46, 47, 49).

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